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TERMINAL PROGRESS REPORT

When this approach to the study of human tumor associated antigens was originally conceived by the P.I. there were questions with regard to its practicality. Whether the proposed approaches would work depended on the answer to two basic questions.

1. Is it possible to obtain large enough quantities of antisera against human tumor associated antigen that is specific enough and strong enough to carry out the proposed work?

2. Are there tumor associated antigens expressed on mouse x human hybrids?

We have provided during this grant period, affirmative answers to these two critical questions:

We were in the process of developing methods to produce good antisera in sufficient quantities when it became clear that the application of the hybridoma system described by Kohler and Milstein to the production of monoclonal antibodies against antigens including cell surface antigens would soon replace the production of conventional antisera and make the analysis of leukemia specific antigens both easier and more definitive. For this reason we have put a major effort into developing the capacity to produce and characterize hybridomas so that now we are in a position to be able to apply it effectively to the analysis of human leukemia specific antigens. With the combination of immunological techniques and somatic cell hybridization available in my laboratory we have been able to effectively develop the hybridoma system and in collaboration with several investigators we have made hybridomas and have produced monoclonal antibodies against a wide variety of antigens (Table 1).

Our general approach to production of monoclonal antibodies against human cell surface antigens has been to use immunization protocols which restrict the response to a limited number of human cell surface antigens (Table 2). Some of these methods were being used in my laboratory for the production of mouse antisera against human cell surface antigens and were applied to the hybridoma production with the intention that restriction of the response to a collection of antigens as complex as that on the surface of human cells would reduce the number of clones that had to be screened to detect a hybridoma making antibody against a particular determinat. Using the procedure of immunizing with cell culture supernatant we have obtained monoclonal antibodies reacting with human B cells (Figure 1, Table 3).

EXHIBIT C

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One of these anti B cell hybridomas was sent to Dr. Donald Capra. It was determined in his laboratory that this antibody reacts with human Ia antigens (p28, p34 proteins) and he is using this antibody to purify human Ia antigens for amino acid sequencing.

A second series of hybridomas was combined from a mouse immunized with a human neuroblastoma IMR6 precoated with a high titered antiserum against a human B cell line. From this fusion we obtained a clone making antibody reacting with human neuroblastomas, a retinoblastoma and fetal brain but exhibiting no detectable activity against other normal human cell types including adult human brain (Figure 2) (see attached reprint). We have found that this oncofetal antigen is expressed on some leukemias and lymphomas (Table 4) although it is not detectable on normal fetal adult bone marrow or peripheral blood cells (see enclosed preprint).

This antibody has also been useful for detection of tumor cells which have metastasized to bone marrow. Using this antibody plus peroxidase linked rabbit anti-mouse immunoglobulin, we have detected neuroblastomas in a patient's bone marrow when they were not detectable with standard clinical screening procedures which are dependent upon the detection of clumps of tumor cells in the marrow (Fig. 3).

Using a similar immunization protocol we have obtained hybridomas making antibodies against leukemia associated antigens (Table 5). During this preliminary period we also began to make mouse x human hybrids with leukemia cells. We made a series of hybrids between PG19, a mouse melanoma cell line, and a null cell acute lymphocytic leukemia (null ALL) received from Dr. Mel Greaves. These hybrids were tested with an anti-null ALL antiserum we had produced plus anti HLA and anti β_2 microglobulin sera. HLA and β_2 could be detected on some clones but there was no detectable expression of the ALL antigen(s). The lack of expression could be attributed to two conditions. (1) The ALL antigen(s) may be lymphoid differentiation antigens not expressed on the background of the mouse melanoma line, (2) the fusion takes place preferentially with cells in the peripheral blood other than blast cells (85% blasts were present).

At this point it was decided that future fusions would be done with mouse lines closely related to the human leukemia using PEG as a fusing agent. Before PEG fusion such a combination would not have been practical. Also there are now available data on the expression of leukemia specific antigens on a series of lymphoid lines derived from leukemia patients. We have obtained several of these lines (Table 6). For most of the mouse x human fusions these lines will be used and thus provide a more homogeneous population of cells to fuse with a closely related mouse cell line. Most of the lines received had mycoplasma and we have treated them with a combination of Tylosine and spectinomycin followed by growth without antibiotics to assure the absence of mycoplasma. Treatment of the lines REH and NALM-1, have resulted in the derivation of mycoplasma-free lines which are now available for fusions.

In collaboration with Dr. Peter Nowell and Dr. D. Rowlands we have, before the cell lines were available, hybridized CLL cells from two different patients with the same plasmacytoma P3-X63Ag8. These hybrids are being used to analyze the expression of CLL immunoglobulin and CLL associated antigens (Table 7).

As indicated in this table some clones of these mouse human hybrids express:

1. Human HLA detected with monoclonal anti-HLA antibodies.
2. The human oncofetal antigen which is expressed on the CLL cells used for fusion (Table 4).
3. The human leukemia associated antigen produced by immunization with the CLL cells used for this fusion.
4. Ia antigens detected by our monoclonal anti human Ia.

Since in this preliminary work, monoclonal antibodies have already been obtained and their expression on mouse x human hybrids detected, it is clear that further work in this area is practical. The antibodies can now be used to isolate the antigens detected. This isolated antigen can then be used to immunize mice and to produce more monoclonal antibodies against other determinants on the same molecule. These antibodies can then be used to detect expression of the antigen in recombinant DNA clones and thus provide a way toward the analysis of the structure of the genes for these antigens.

Reprints and preprints of work under this grant are attached.

TABLE 1

FUSIONS WITH MOUSE MYELOMAS FROM WHICH WE HAVE ISOLATED CLONED ANTIBODY PRODUCING LINES

ANTIGEN		COLLABORATORS
DNP NP PC GLA5	Adult spleen cells Neonatal spleen cells + LPS Monoclonal spleen fragments	K. Denis, N. Klinman, A. Tung P. Stashenko, C. Merryman
Mouse Testes, Mouse Brain and Neuroblastoma C1300		K. Bechtol
Mouse Lymphoma L5178Y		L. Manson
Ag-B		D. Gasser
Human Alkaline Phosphatase		C. Slaughter, M. Cancro
Human Neuroblastoma		F. Gilbert
Human ALL (Null)		M. Greaves
Human CLL (B)		P. Nowell, D. Rowlands
Human B Cell (concentrated culture supernatant)		
Streptococcal Type Specific Antigens		R. Polin
Hamster Dihydrofolate Reductase		R. Schoner, J. Littlefield

TABLE 2

Methods for Restricting Heterospecific Response to Human Cell Surface Antigens

Method	Reference
Immunization with purified antigen-applicable to well characterized antigens such as HLA, β_2 microglobulin.	(Goodfellow et al., 1976)
Immunization with cells coated with antibodies made against another human cell type.	(Brown et al., 1975)
Immunization with cell culture supernatant.	(Kennett et al., 1978)
Immunization with mouse x human hybrids having only a few human chromosomes and thus expressing a limited number of human antigens.	(Buck and Bodmer, 1975)
Identification of monoclonal spleen fragments making antibody against human alloantigens. As shown in (Kennett et al., 1978) these fragments can be used for hybridoma production.	(Lampson et al., 1978)
Immunization of primates with human cells. Hybridization of the primate spleen or lymph node cells may thus result in production of primate antibodies against specific human antigens.	(Metzger et al., 1972)

TABLE 3 Reactivity of P3B Hybridoma Supernatants with Human Cells

Hybridoma Supernatant	P3BA2 P359B	P3B34 P3BA1 P3B84	P3B13C2/5 P3B63	P3B1C3	PI153/3
BJAB, B (EB-)	+	+	+	+	-
8866, B	+	+	+	+	-
McGiffert[21], B	+	+	+	+	-
SCBM, B	+	+	+	+	-
Daudi, B ($-\beta_2$, HLA)	+	-	+	-	-
MOLT 4, T	+	+	-	-	-
REH, Null	+	+	+	+	-
SKHep[23], Hepatoma	+	+	-	+	-
IMR 6[22] Neuroblastoma	+	+	-	-	+
HT1080[25] Fibrosarcoma	+	+	-	-	-
D98 AH/2[26] HeLa	+	+	-	-	-
Thymus	+	+	-	-	-
Peripheral Blood Lymphocytes	+	+	20-30*	+	-
Fibroblasts	+	+	-	-	-

Table 3 Reactivity of P3B hybridoma supernatants against a panel of human cell types. Binding of antibody to the cells was detected with ^{125}I rabbit anti mouse Fab as indicated in Figure 1. Background counts were less than 1% of input counts. + indicates at least 2X background counts. PI153/3 is a hybridoma supernatant reacting specifically with human neuroblastomas. Reactivity of P3B13C2/5 and P3B1C3 against peripheral lymphocytes was determined by microcytotoxicity against a panel of 20 HLA (A,B) typed individuals. The indicated reaction was the same for each person tested.

The clones P3B1C3 and P3B13C2 were passaged as ascites tumors in pristane primed mice.[7] The supernatants from these hybrids showed significant binding to cells at dilutions of 1/32 and 1/128 whereas the activity in the ascites fluids had binding titers of 1/4000.

TABLE 4

EXPRESSION OF DIFFERENTIATION ANTIGENS DETECTED BY MONOCLONAL ANTIBODIES ON MOUSE x HUMAN HYBRIDS

CELL LINE	PI153/3 Oncofetal Antigen	P3B13C2 Anti B-cell	H76A2 Anti B-cell	W6/32 HLA
IMR6 - neuroblastoma	+			+
8866 - B cell line		+	+	+
D-CLL - Chronic Lymphocytic Leukemia	+	+	+	+
Neuroblastoma x fibroblast hybrids:				
NRSTP-4M				
NRSTP-76	+			
N48TP-8				
Leukemia x plasmacytoma hybrids:				
P3D 13			+	+
P3D 18	+		+	+
P3D 19			+	+
P3D 20				
P39 30		+	+	+
P3D 32	+		+	+

Table 4

The expression of antigens identified by four monoclonal antibodies were detected on mouse x human hybrids by cytotoxicity and immunoflourescence. PI153/3 reacts with neuroblastomas, fetal brain, and some leukemias; P3B13C2 with human B-cells, H76A2 with human, mouse and rat B cells (see preprint) and W6/32 against HLA (Parham et al., 1978). The H76A2 and W6/32 (HLA) antigens are on the same chromosome (#6) while the other two antigens are on two other chromosomes.

TABLE 5

Antibodies Against Human Leukemia Associated Antigens

	B-CLL	REH null ALL	B-cell line	T-cell line	HT1080 Fibrosarcoma	SKHep Hepatoma	Peripheral Blood Lymphocytes
PAD 10	+	+	-	-	-	-	-
PAD 28	+	+	-	-	-	-	-
SPAR 8/1	-	+	-	-	-	-	-

Table 5 Reactions of monoclonal anti-leukemia antibodies with various human cell types. PAD hybridomas were made with spleen cells from mice immunized with a human B-CLL coated with an antihuman antiserum. SPAR hybridomas were made from spleen cells from mice immunized with REH a human null ALL cell line. They were selected by screening first against the immunizing cell and then against a panel of other non leukemia cells. They are either against leukemia specific antigens or differentiation antigens present in the leukemia cells and a mini subpopulation of human lymphocytes.

Tests were done by microcytotoxicity and by binding assays.

TABLE 6

Human Leukemia and Burkitt's Lines Tested for Leukemic Antigens

	Origin	Cell types	CALL	HTLA
NALM-1	CML	null	+	
CCRF-CEM	ALL	T	+	+
RPMI 8402	ALL	T	+	+
MOLT 4	ALL	T		-
Daudi	Burkitt's	B	+	-
HRIK	Burkitt's	B	-	-
Raji	Burkitt's	B	-	-
REH	ALL	null	+	

Table 6 Leukemia cell lines and tumor lines tested for Common Acute Lymphocytic Leukemia antigen (CALL) and human Thymus Leukemia antigen (HTLA) (Minowada et al., 1978). These lines have been obtained and are available as a screening panel and for mouse x human hybridizations. We also have available nearly a hundred other B lymphoblastoid lines many of which are defined with regard to HLA A, B, and D specificities.

TABLE 7

Type of Leukemia	Source	Other types of antibodies that that may be obtained
B-CLL	P. Nowell-large numbers of these cells can be obtained from a patient for several years.	anti B cell, anti Ig, anti HLA
T-CLL	D. Rowlands-cells may be obtained from same patient for several years.	anti T cell subset, anti T receptor, anti HLA
Null ALL	NULL ALL lines	anti Ia, null cell specific, anti HLA

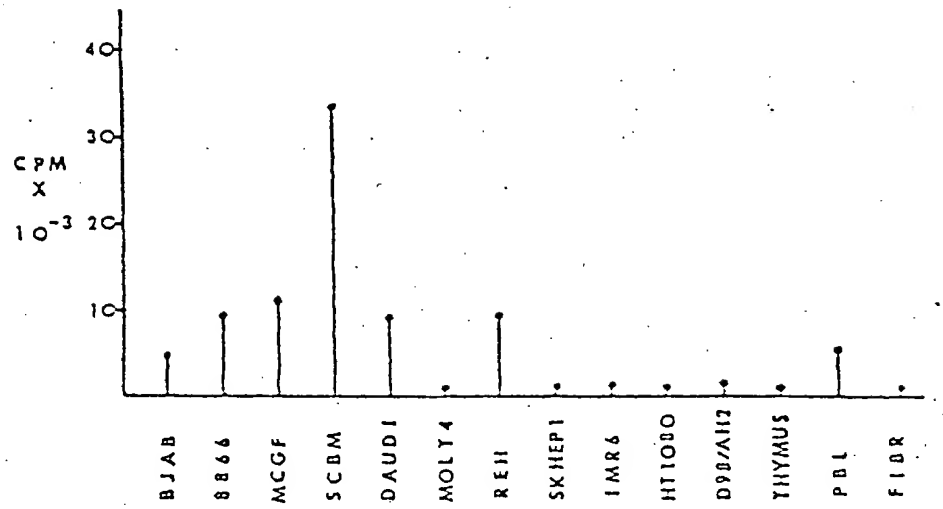

Figure 1 Binding of monoclonal antibody P3B13C2/5 against a panel of human cell types. Hybridomas were made by fusing P3x63Ag8 with spleen cells from mice immunized with B lymphoblastoid line culture supernatant (see attached preprint).

0.5-1 x 10^6 of each cell type was incubated for 1 hour at 4°C with 25 µl of supernatant. The cells were washed and ^{125}I anti-mouse Fab added (100,000 cpm). After incubation for 1 hour at 4°C the tubes were washed twice and counted. Samples were done in duplicate.

Control supernatants from P3/X63-Ag8 (γ_1) and from RK4146 (µ), hybridoma which produces anti-DNP antibody, did not bind significantly to any of the cell types tested. PI 153 (µ), a hybridoma producing antibody against human neuroblastomas used as a control, bound only to neuroblastomas and not to any of the other human cell types (Kennett and Gilbert, in preparation). B cell lines: BJAB (EBV-), 8866, McGiffert (MCGF) Daudi, REH, null cell line; MOLT 4, T cell line; SKHep-1, hepatoma; IMR6, neuroblastoma; D98/AH2, HeLa; Thymus, adult thymus cells, PBL, peripheral blood lymphocytes; FIBR, diploid fibroblasts.

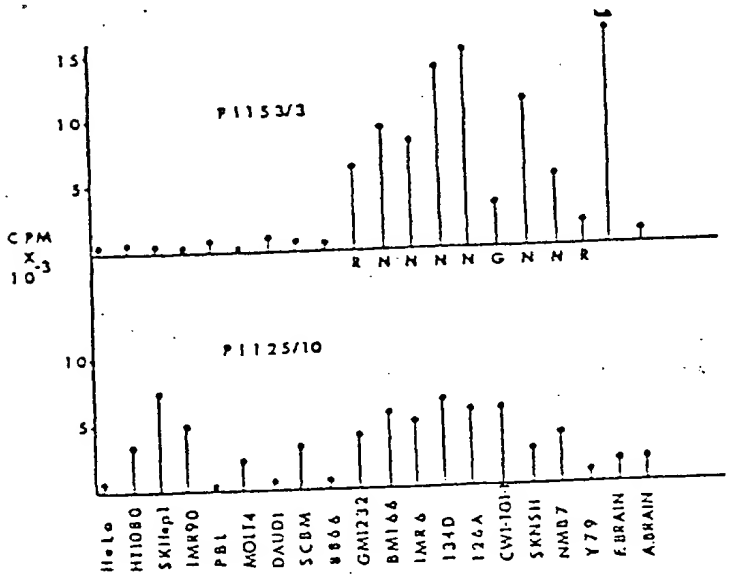

Figure 2a Binding of PI153/3 and PI125/10 antibodies to human cells. 0.5-1 x 10^6 of each cell type or an equivalent packed cell volume of homogenized brain were incubated for 1 hour at 4°C with 25 µl of supernatant or diluted ascites fluid. The cells were washed and ^{125}I anti-mouse Fab added (100,000 cpm). After incubation for 1 hour at 4°C the tubes were washed twice and counted. Samples were done in duplicate. Neuroblastomas: (N) 134D, IMR6, 126A, BM1-6, SKNSH, NMB7; Retinoblastomas: (R) GM1232, Y79; Glioblastoma: (G) CW1-TG1-1 (A. McMorris, Wistar Institute); Fibrosarcoma: HT1090 (C. Croce, Wistar Institute); Hepatoma: SKHep-1 (J. Fogh, Sloan Kettering); Fibroblast: IMR90 (Institute for Medical Research, Camden); B Lymphoblastoid Lines: Daudi, SCBM, 8866; T Cell Line: MOLT4; Peripheral Blood Lymphocytes: pbl; HeLa: D98-AH/2. The same background binding is found with whole adult human brain or with separate preparations of grey or white matter from cerebellum or cerebrum.

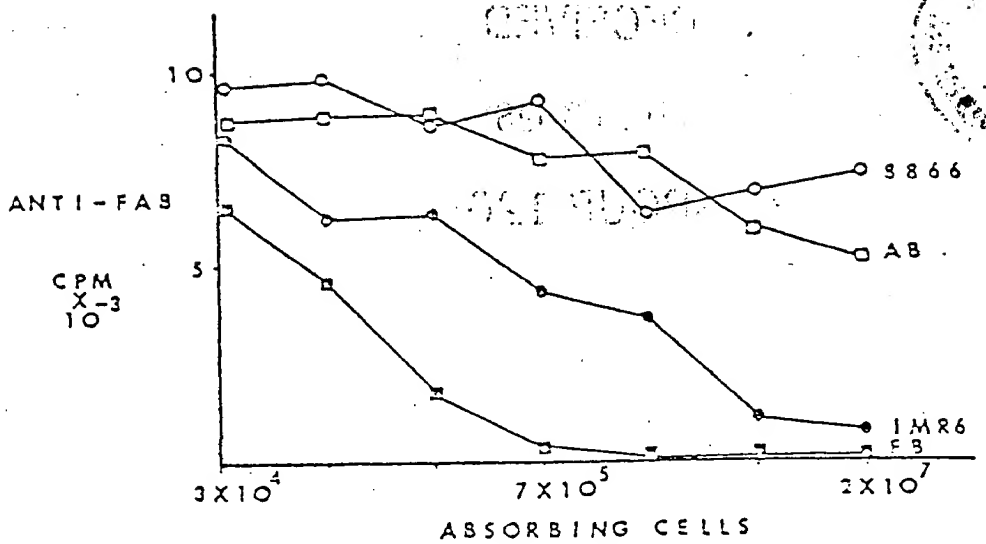

Figure 2b Absorption of PI153/3 ascites. PI153/3 ascites (1/100) was absorbed with 8866 a B lymphoblastoid line IMR6 a neuroblastoma and packed cell equivalents of homogenized human adult brain and fetal brain and the binding of the absorbed antibody solution tested against IMR6. The indicated number of cells (1/3 dilutions) suspended in a final volume of 50 µl was incubated with 50 µl of ascites fluid at a dilution of 1/1000 for 1 hour at 4°C. The cells were removed by centrifugation and the antibody remaining in the supernatant detected by binding to IMR6 using the binding assay described in Figure 1. The ratio of packed cell volume to liquid at the highest concentration of cells was 1/2. 8866 (O); IMR6 (●); Adult brain □ (AB); Fetal brain ▭ (FB). The antigen detected by PI153/3 antibody is detected in high concentration on IMR6 and fetal brain but there is little if any on adult brain and 8866. Assuming the reduction in titer with adult brain is due to the presence of the antigen and not a dilution effect the relative amounts on adult brain, neuroblastoma and fetal brain is 1:26:190.